



Year: 2021

Altered CSF Albumin Quotient Links Peripheral Inflammation and Brain Damage in MS

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Abstract: OBJECTIVE CNS damage can increase the susceptibility of the blood-brain barrier (BBB) to changes induced by systemic inflammation. The aim of this study is to better understand BBB permeability in patients with MS and to examine whether compromised BBB integrity in some of these patients is associated with CNS damage and systemic inflammation. METHODS Routine CSF measurements of 121 patients with MS were analyzed including number and type of infiltrating cells, total protein, lactate, and oligoclonal bands, as well as intrathecal production of immunoglobulins and CSF/serum quotients for albumin, immunoglobulins, and glucose. In addition, in a subcohort of these patients, we performed ex vivo immunophenotyping of CSF-infiltrating and paired circulating lymphocytes using a panel of 13 monoclonal antibodies, we quantified intrathecal neurofilament light chain (NF-L) and chitinase 3-like 1 (CHI3L1), and we performed intrathecal lipidomic analysis. RESULTS Patients with MS with abnormal high levels of albumin in the CSF showed a distinct CSF cell infiltrate and markers of CNS damage such as increased intrathecal levels of NF-L and CHI3L1 as well as a distinct CSF lipidomic profile. In addition, these patients showed higher numbers of circulating proinflammatory Th1 and Th1* cells compatible with systemic inflammation. Of interest, the abnormally high levels of albumin in the CSF of those patients were preserved over time. CONCLUSIONS Our results support the hypothesis that CNS damage may increase BBB vulnerability to systemic inflammation in a subset of patients and thus contribute to disease heterogeneity.

DOI: <https://doi.org/10.1212/NXI.0000000000000951>

Posted at the Zurich Open Repository and Archive, University of Zurich

ZORA URL: <https://doi.org/10.5167/uzh-205256>

Journal Article

Published Version



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Originally published at:

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Mireia (2021). Altered CSF Albumin Quotient Links Peripheral Inflammation and Brain Damage in MS. *Neurology: Neuroimmunology and Neuroinflammation*, 8(2):e951.
DOI: <https://doi.org/10.1212/NXI.0000000000000951>

Altered CSF Albumin Quotient Links Peripheral Inflammation and Brain Damage in MS

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Neurol Neuroimmunol Neuroinflamm 2021;8:e951. doi:10.1212/NXI.0000000000000951

Abstract

Objective

CNS damage can increase the susceptibility of the blood-brain barrier (BBB) to changes induced by systemic inflammation. The aim of this study is to better understand BBB permeability in patients with MS and to examine whether compromised BBB integrity in some of these patients is associated with CNS damage and systemic inflammation.

Methods

Routine CSF measurements of 121 patients with MS were analyzed including number and type of infiltrating cells, total protein, lactate, and oligoclonal bands, as well as intrathecal production of immunoglobulins and CSF/serum quotients for albumin, immunoglobulins, and glucose. In addition, in a subcohort of these patients, we performed ex vivo immunophenotyping of CSF-infiltrating and paired circulating lymphocytes using a panel of 13 monoclonal antibodies, we quantified intrathecal neurofilament light chain (NF-L) and chitinase 3-like 1 (CHI3L1), and we performed intrathecal lipidomic analysis.

Results

Patients with MS with abnormal high levels of albumin in the CSF showed a distinct CSF cell infiltrate and markers of CNS damage such as increased intrathecal levels of NF-L and CHI3L1 as well as a distinct CSF lipidomic profile. In addition, these patients showed higher numbers of circulating proinflammatory Th1 and Th1* cells compatible with systemic inflammation. Of interest, the abnormally high levels of albumin in the CSF of those patients were preserved over time.

Conclusions

Our results support the hypothesis that CNS damage may increase BBB vulnerability to systemic inflammation in a subset of patients and thus contribute to disease heterogeneity.

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Go to [Neurology.org/NN](https://www.neurology.org/NN) for full disclosures. Funding information is provided at the end of the article.

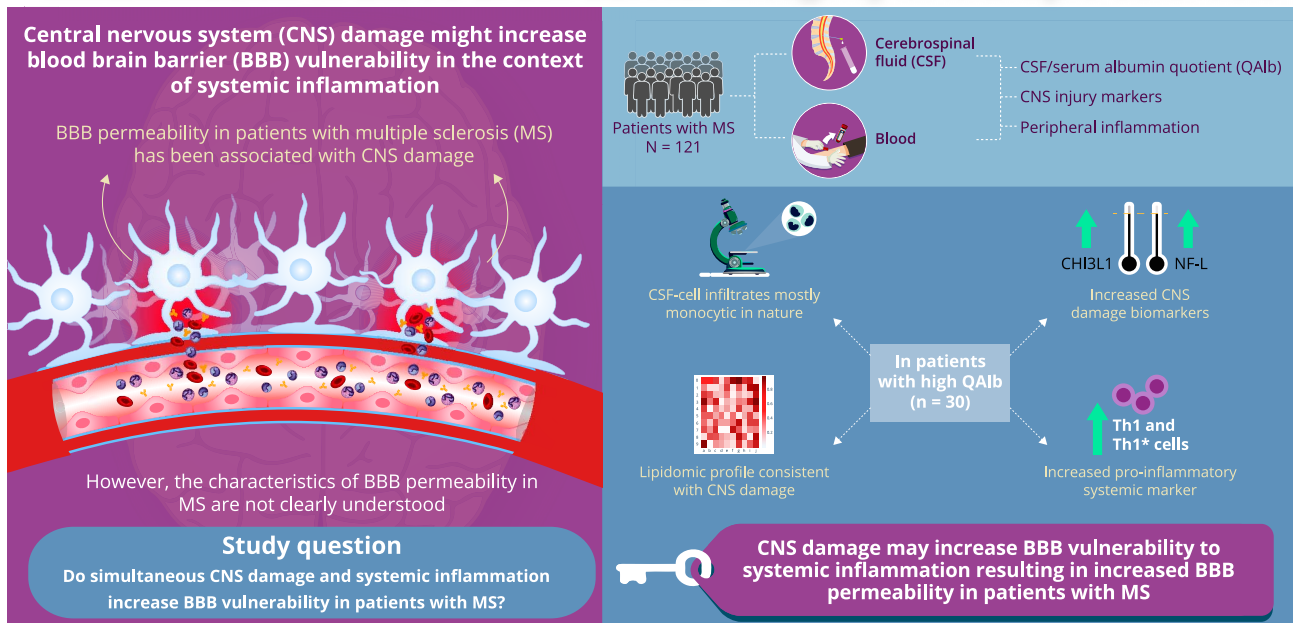
The Article Processing Charge was funded by the authors.

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Glossary

BBB = blood-brain barrier; **CHI3L1** = chitinase 3-like 1; **FADS3** = fatty acid desaturase 3; **HLA** = human leukocyte antigen; **IFN** = interferon; **Ig** = immunoglobulin; **NF-L** = neurofilament light chain; **OIND** = other inflammatory neurologic diseases; **ONIND** = other noninflammatory neurologic diseases; **PC** = phosphatidylcholine; **QAlb** = serum albumin quotient; **QNorm** = normal QAlb; **RBC** = red blood cell; **SAdienine** = sphingadienine; **SM** = sphingomyelin.

Role of Central Nervous System Damage and Systemic Inflammation in Blood Brain Barrier Integrity in Multiple Sclerosis



doi:10.1212/NXI.0000000000000951

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Neurology
Neuroimmunology
& Neuroinflammation

Systemic inflammation can influence blood-brain barrier (BBB) permeability and increase vesicular trafficking of albumin from serum to the CNS.¹ In animal models of Alzheimer disease,² ischemic stroke,³ and MS,^{4,5} it has been observed that the effect of systemic inflammation on BBB permeability was higher in animals with these CNS pathologies, suggesting that brain damage could increase the susceptibility of the BBB to changes induced by systemic inflammation. Consistent with these findings, a human study demonstrated a link between markers of systemic inflammation and markers of increased BBB permeability, in particular high CSF/serum albumin quotient (QAlb) in individuals with abnormal CSF measures but not in those without. Because abnormal CSF measures most likely reflect CNS alterations, these results support a role of CNS damage in increasing BBB susceptibility to systemic inflammation.⁶

MS is a heterogeneous immune-mediated neurologic disease that damages the CNS. This damage might increase the susceptibility of the BBB to changes induced by a systemic inflammation in some patients and contribute to MS heterogeneity. Supporting such an association between CNS

damage and changes in BBB permeability, it has been observed that high QAlb and other CSF markers of compromised BBB integrity in patients with MS are accompanied by brain atrophy and faster increase in disability.^{7,8}

To better understand BBB permeability in MS and its putative association with CNS damage and systemic inflammation, we performed a detailed characterization of CSF and paired blood samples that included immunophenotyping and lipidomics.

Methods

Patient Material

Paired CSF and blood samples were collected from 121 untreated patients with MS (20 patients with radiologically or clinically isolated syndrome, 91 with relapsing-remitting MS, and 10 with progressive MS), 40 controls with other non-inflammatory neurologic diseases (ONIND), and 21 with other inflammatory neurologic diseases (OIND). All CSF samples were obtained for diagnostic purposes. Demographic

Table 1 Demographic and Clinical Features of Patients With MS and Controls

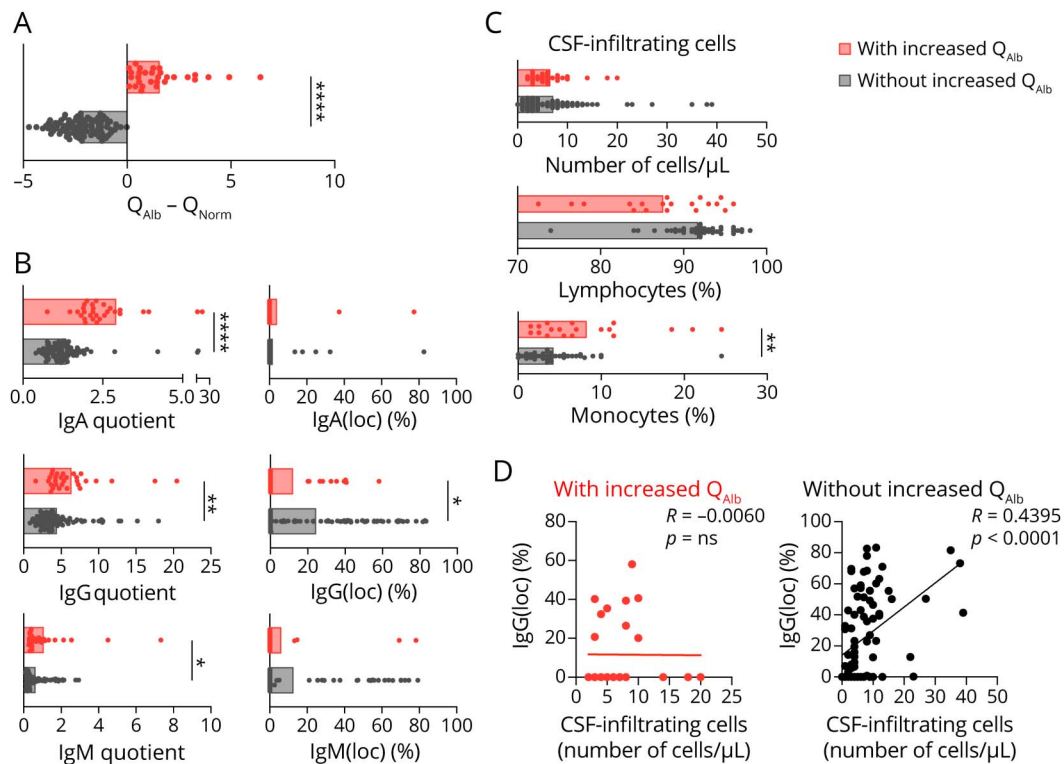
	MS cohort			Controls	
	Main	Sub-1	Sub-2	ONIND	OIND
No. of patients	121	79	36	40	21
Female/male ratio	2.02	2.16	2.3	0.72	0.72
Age (y)	35.9 ± 9.9	35.9 ± 10.2	37.5 ± 11.4	40.7 ± 12.6	45.2 ± 9.5
Disease duration (mo)	25.5 ± 48.3	26 ± 51.7	29.4 ± 55.1		
	MS main cohort				
	With increased QAlb		Without increased QAlb	p Value	
No. of patients	30		91		
Female/male ratio	1.1		2.5		
Age at CSF puncture (y)	35.2 ± 10.2		36.6 ± 10	ns	
Age at onset (y)	32.1 ± 8.3		34.4 ± 9.8	ns	
RIS/CIS (%)	33.4		40		
RRMS (%)	56.6		53.7		
PMS (%)	10		6.3		
Disease duration (mo)	27.6 ± 49.2		26.5 ± 48.3	ns	
Time since last relapse (d)	119.3 ± 317.7		287.8 ± 642.5		
Clinically active ^a (%)	73.3		54.9		
Gd contrast-enhancing lesions ^a (%)	60		48.7		
Lesions location					
Optic neuritis (%)	28.6		18.6		
Myelitis (%)	25		19.7		
Brainstem (%)	7.1		8.1		
Several (%)	39.3		53.6		
Expanded Disability Status Scale	2.25 ± 1.8		1.7 ± 1.5		
HLADR15 (%)	41.3		50.5		
CSF					
CSF OCB type II (%)	90		81		
Albumin quotient	7.94 ± 1.03		4.24 ± 1.81	<0.0001	
IgA index	0.37 ± 0.36		0.32 ± 0.27	ns	
IgG index	0.79 ± 0.38		1.11 ± 0.87	ns	
IgM index	0.14 ± 0.21		0.16 ± 0.16	ns	
Patients with IgG(loc) >10% (%)	35.7		52.9		
Total intrathecal protein (mg/L)	619.0 ± 159.7		392.8 ± 88.88	<0.0001	
Intrathecal lactate (mmol/L)	1.64 ± 0.20		1.61 ± 0.25	ns	
Glucose quotient	0.65 ± 0.09		0.66 ± 0.11	ns	
Albumin in serum (g/L)	46.08 ± 3.57		45.63 ± 5.03	ns	

Abbreviations: CIS = clinically isolated syndrome; Ig = immunoglobulin; OCB = oligoclonal band; OIND = other inflammatory neurologic diseases; ONIND = other noninflammatory neurologic diseases; PMS = progressive MS; QAlb = serum albumin quotient; QNorm = normal QAlb; RIS = radiologically isolated syndrome; RRMS = relapsing-remitting MS.

Mean ± SD is shown.

^a Clinically active means during a relapse, i.e., in the presence of new neurologic symptoms lasting at least 24 hours and with no evidence of signs/symptoms of systemic inflammation within 3 months before or after lumbar puncture.

Figure 1 Characterization of CSF From Patients With MS With and Without Increased Q_{Alb}



(A) Graph shows Q_{Alb} minus Q_{Norm} . Patients in which this difference is positive are shown in red and those in which is negative in black. Comparison between patients with and without increased Q_{Alb} of: (B) quotients and Igs(loc) expressed as percentage (%) for IgA, IgG, and IgM and (C) CSF-infiltrating cells (number of cells/ μ L) and frequency (%) of lymphocytes and monocytes analyzed under the microscope. (D) Correlation between number of CSF-infiltrating cells and IgG(loc) expressed as percentage (%). Graphs (A–D) represent the main cohort of 121 patients with MS. Each dot in the graphs corresponds to 1 patient, and bars show the mean. A t test for normally distributed variables and U test (Mann-Whitney) for non-normally distributed variables was used to compare patients. Linear correlation was tested using Pearson for normally distributed and Spearman r for non-normally distributed variables. Statistical significance (* $p < 0.05$, ** $p < 0.01$, and **** $p < 0.0001$) is shown. Ig = immunoglobulin; Q_{Alb} = serum albumin quotient; Q_{Norm} = normal Q_{Alb} .

and clinical characteristics are summarized in table 1. Patients were recruited from the Neuroimmunology and MS Research Section, Neurology Clinic, University Hospital Zurich. MS diagnosis was based on the revised McDonald criteria.⁹ Patients who had not received steroids at least 4 weeks before enrollment or any immunomodulatory or immunosuppressive agent during the last 3 months were considered untreated and included in the study. In particular, 97 patients were never treated, 16 patients were previously treated only with steroids, 4 patients received steroids and an immunomodulatory or immunosuppressive agent (1 patient, interferon [IFN] β -1a; 1 patient, glatiramer acetate; and 2 patients, dimethyl fumarate), and 4 patients received only an immunomodulatory or immunosuppressive agent (1 patient, IFN β -1a; 2 patients, glatiramer acetate; and 1 patient, dimethyl fumarate).

Standard Protocol Approvals, Registrations, and Patient Consents

The Cantonal Ethics Committee of Zurich approved the study procedures (EC-No. 2013-0001). Informed consent was obtained from all patients or relatives.

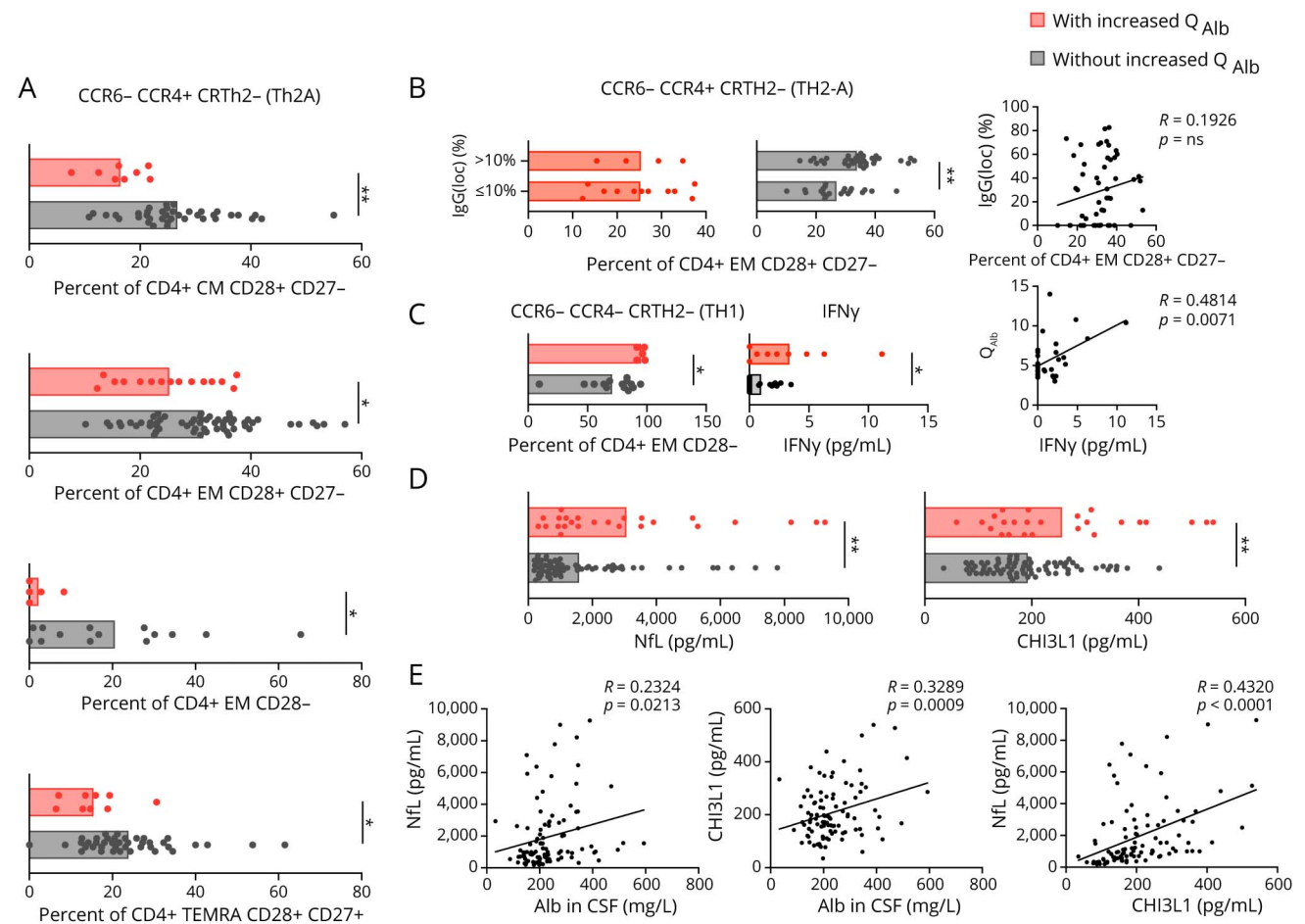
Routine CSF and Serum Measures

CSF-infiltrating cells were counted with a Fuchs Rosenthal counting chamber under the microscope within 1 hour after lumbar puncture. CSF total protein as well as CSF and serum albumin, immunoglobulin (Ig) G, IgM, and IgA were determined by immunonephelometry. Quotients (Q) were defined as $Q = (\text{concentration in CSF [mg/L]} / \text{concentration in serum [g/L]})$. Because Q_{Alb} increases with age, we calculated a maximum normal Q_{Alb} (Q_{Norm}) for each patient that takes into account the age at lumbar puncture ($Q_{Norm} = [\text{age}/15] + 4 \times 10^{-3}$).¹⁰ Intrathecal Ig synthesis (Ig(loc)) was calculated using hyperbolic functions according to Reiber and expressed as percentage (%).¹¹ Identification of CSF-restricted oligoclonal bands was performed by isoelectric focusing.

HLA Typing

Patients were typed for human leukocyte antigen (HLA) class I (A* and B*) and II (DRB1*, DRB3*, DRB4*, DRB5*, DQA1* and DQB1*) molecules using high-resolution HLA sequence-based typing at Histogenetics LLC, Ossining, NY. Isolation of DNA from whole blood with a final concentration of 15 ng/ μ L was performed with a standard DNA isolation

Figure 2 Characterization of CSF-Infiltrating Cells and Markers of CNS Damage in Patients With MS With and Without Increased Q_{Alb}



(A) Comparison between patients with and without increased Q_{Alb} of frequencies of Th2-A (CCR6- CCR4+ CRTH2-) in CM CD28⁺ CD27-, EM CD28⁺ CD27-, EM CD28⁺ CD27- CD4⁺ CSF-infiltrating T cells. (B) Comparison between patients with IgG(oc) (>10%) and without IgG(oc) (<10%) of frequencies of EM CD28⁺ CD27- CD4⁺ CSF-infiltrating cells with a Th2-A (CCR6- CCR4+ CRTH2-) functional phenotype. Correlation between these frequencies and IgG(oc) expressed as %. (C) Comparison between patients with and without increased Q_{Alb} of frequencies of Th1 (CCR6- CCR4- CRTH2-) cells in EM CD28- CD4⁺ CSF-infiltrating T cells and amount of IFN γ expressed as pg/mL present in the CSF of 30 patients as well as correlation between the amount of IFN γ and Q_{Alb}. (D) Comparison of NF-L and CHI3L1 intrathecal concentration between patients with and without increased Q_{Alb}. (E) Correlation between the intrathecal concentration of these 2 biomarkers and Q_{Alb} and also between themselves. Graphs (A–C) represent subcohort 1 of 79 patients with MS, and frequencies have been obtained by ex vivo immunophenotyping, and (E–D) represent the main cohort of 121 patients with MS. Each dot in the graphs corresponds to 1 patient, and bars show the mean. Patients with increased Q_{Alb} are shown in red. T test t test for normally distributed variables and U test (Mann-Whitney) for non-normally distributed variables was used to compared patients. Linear correlation was tested using Pearson for normally distributed and Spearman r for non-normally distributed variables. Statistical significance (* $p < 0.05$, ** $p < 0.01$) is shown.

protocol using a Triton lysis buffer and proteinase K treatment.

Flow Cytometric Immunophenotyping

Flow cytometric immunophenotyping of CSF-infiltrating and paired circulating lymphocytes was performed as previously reported.¹² Briefly, a minimum of 10,000 CSF-infiltrating cells were concentrated by centrifugation and processed in the first hour after collection. Blood circulating cells were obtained from 800 μ L of peripheral blood after lysis of red blood cells (RBCs) using RBC Lysis buffer (BioLegend, San Diego, CA). Cells were stained with a cocktail of 13 monoclonal antibodies.¹²

SPHERO AccuCount Particles (Sperotech, Inc., Lake Forest, IL) were added to determine absolute counts following

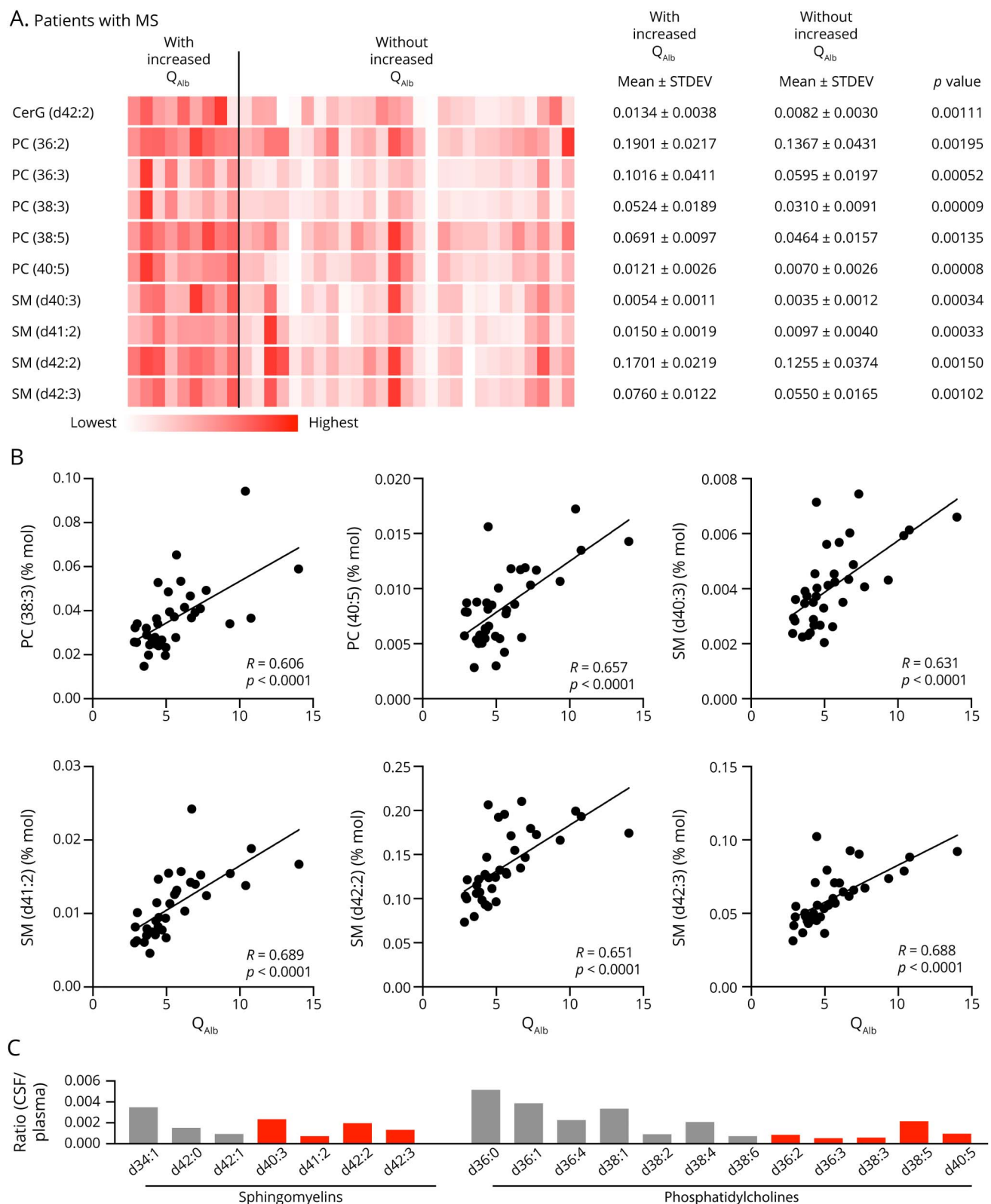
manufacturer's instructions. Sample acquisition was performed in an LSR Fortessa cytometer (BD Biosciences, Franklin Lakes, NJ), and data were analyzed using FACSDiva (BD) and FlowJO (TreeStar Inc., Ashland, OR) software. The gating strategy is summarized in figure e-1 (links.lww.com/NXI/A413).

Legend Plex and ELISAs

Cytokines directly in the CSF were measured using the Human T Helper Cytokine Panel LEGENDplex bead-based immunoassay (BioLegend) according to the manufacturer's instructions.

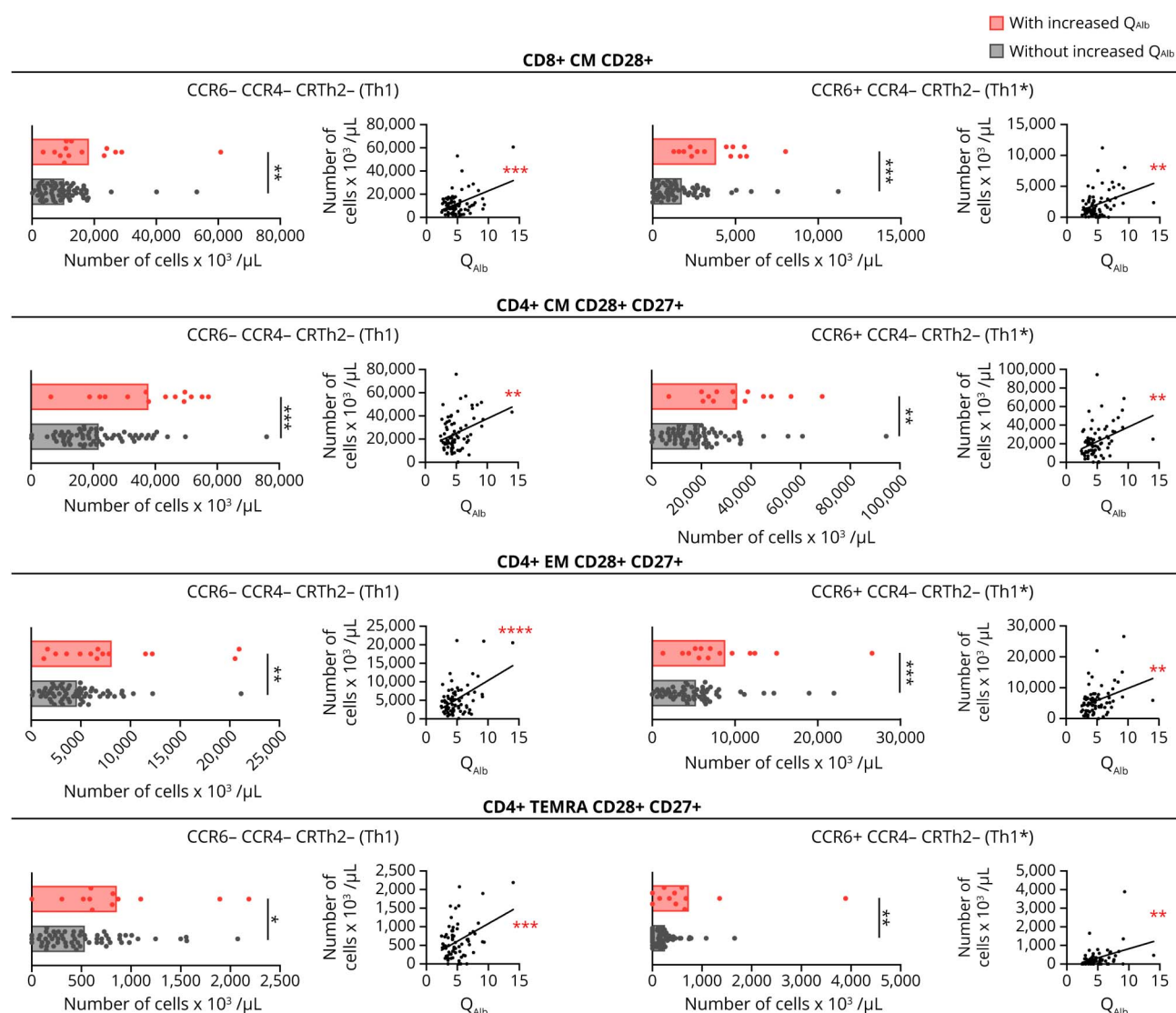
Intrathecal neurofilament light chain (NF-L) and chitinase 3-like 1 (CHI3L1) proteins were quantified in CSF samples

Figure 3 Lipidomic Profile in Patients With MS With and Without Increased Q_{Alb}



(A) Heat map of the 10 statistically most significant altered lipid species between patients with MS with and without increased Q_{Alb} . The abundance of the lipid species is qualitatively represented by color intensity for each of the 36 patients with MS (subcohort 2). Mean, SD, and p values of the group comparisons are shown. (B) Correlations between Q_{Alb} and the intrathecal concentration for 6 of the identified lipid species. Linear correlation between variables was tested using Pearson correlation, and p values are shown. (C) Ratios between mean CSF concentration of lipid species in patients with MS and the concentration in plasma from a reference control cohort with 24 healthy controls. Marked in red are the 4 SM and 5 PC species that were significantly more abundant in patients with MS with increased Q_{Alb} . Ig = immunoglobulin; Q_{Alb} = serum albumin quotient.

Figure 4 Immunophenotyping of Peripheral Circulating Lymphocytes in Patients With MS With and Without Increased Q_{Alb}



Comparison, between patients with (red) and without (black) increased Q_{Alb}, of number of CD8⁺ CM CD28⁺ Th1 (CCR6⁻ CCR4⁻ CRTh2⁻) and Th1* (CCR6⁺ CCR4⁻ CRTh2⁻), CD4⁺ CM CD28⁺ CD27⁺ Th1 (CCR6⁻ CCR4⁻ CRTh2⁻) and Th1* (CCR6⁺ CCR4⁻ CRTh2⁻), CD4⁺ EM CD28⁺ CD27⁺ Th1 (CCR6⁻ CCR4⁻ CRTh2⁻) and Th1* (CCR6⁺ CCR4⁻ CRTh2⁻), and CD4⁺ TEMRA CD28⁺ CD27⁺ Th1 (CCR6⁻ CCR4⁻ CRTh2⁻) and Th1* (CCR6⁺ CCR4⁻ CRTh2⁻) in patients with and without increased Q_{Alb}, as well as the correlation between these cell counts and Q_{Alb}. All graphs represent subcohort 1 consisting of 79 patients with MS. Each dot in the graph corresponds to a single patient, and bars show the mean. T test t test for normally distributed variables and U test (Mann-Whitney) for non-normally distributed variables was used to compared patients. Linear correlation was tested using Pearson r for normally distributed and Spearman r for non-normally distributed variables. Statistical significance (**p* < 0.05, ***p* < 0.01, ****p* < 0.001, and *****p* < 0.0001) is shown. Statistical significance (**p* < 0.05, ***p* < 0.01, ****p* < 0.001, and *****p* < 0.0001) is shown. Q_{Alb} = serum albumin quotient.

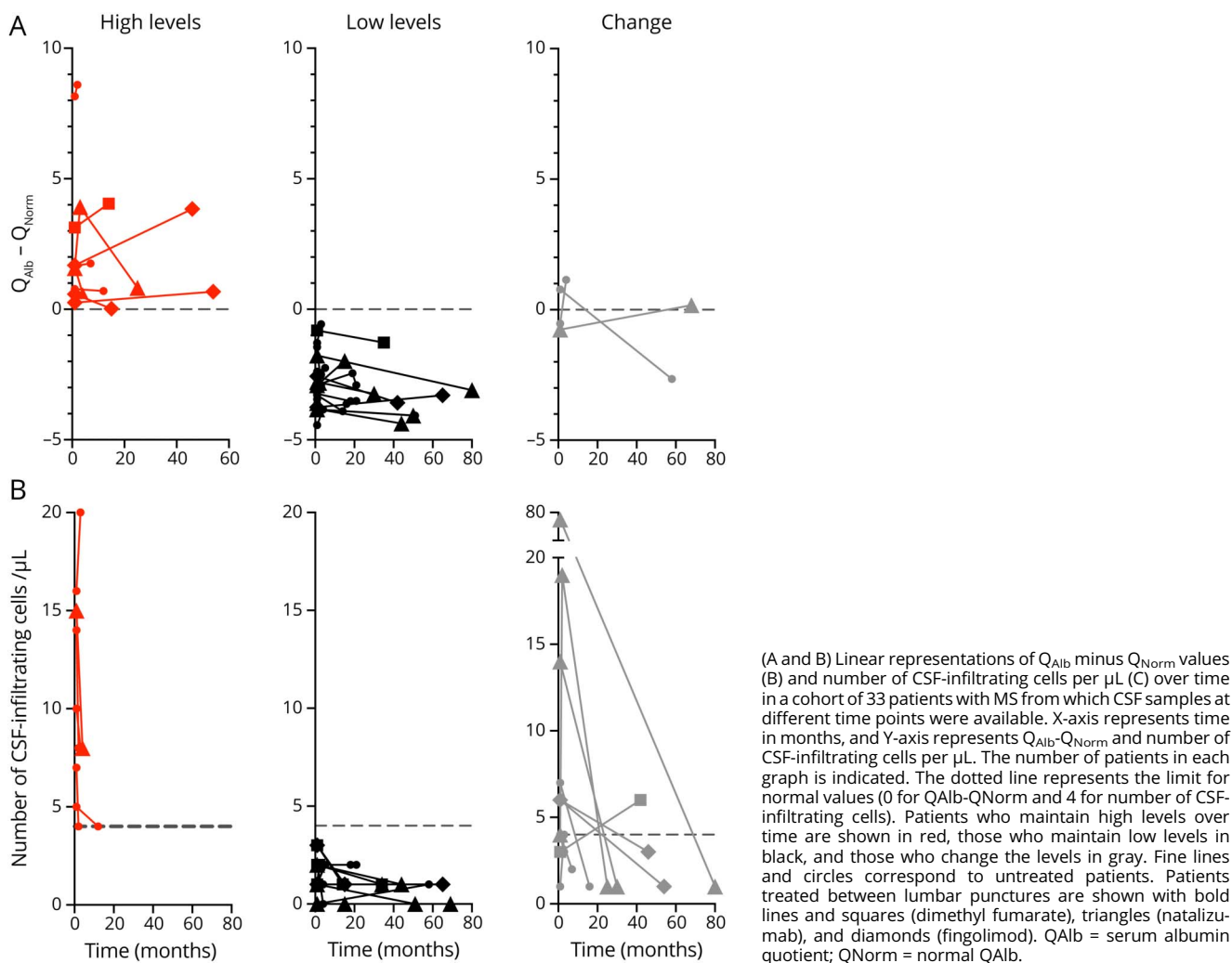
by ELISA (Human Diagnostics, Umea, Sweden, and MicroVue, Athens, OH, respectively) according to the manufacturer's instructions.

Lipid Extraction and Lipidomics

Lipid extraction was performed as previously described¹³ with some modifications. The MMC solvent (methanol: methyl tert-butyl ether: chloroform, 4:3:3, vol:vol:vol) was supplemented with the SPLASH mix internal standard and additional internal standards (all from Avanti Polar Lipids): d7-sphinganine (SPH d18:0), d7-sphingosine (SPH d18:1), dihydroceramide (Cer d18:0/12:0), ceramide

(Cer d18:1/12:0), glucosylceramide (GluCer d18:1/8:0), sphingomyelin (SM d18:1/12:0), and d7-sphingosine-1-phosphate (S1P d18:1). Liquid chromatography was performed according to Ref. 14 with some modifications. Lipids were separated using a C30 Accucore LC column (150 × 2.1 mm, 2.6 μm particle size) and a Transcend UHPLC pump (Thermo Fisher Scientific). Mass spectrometry analysis was performed on a hybrid quadrupole-orbitrap mass spectrometer (Q-Exactive; Thermo Fisher Scientific). Lipid identification criteria were (1) resolution with an accuracy of 5 ppm from the predicted mass at a resolving power of 70,000 at 200 m/z, (2) isotopic

Figure 5 Q_{Alb} Analysis Over Time



distribution, (3) expected retention time, and (4) fragmentation pattern.

Data analysis was performed using TraceFinder 4.1 (Thermo Fisher Scientific) for peak picking, annotation, and matching to an in-house lipid database.

Statistics

For comparison of 2 groups of patients, we used the unpaired *t* test for normally distributed variables and the *U* test (Mann-Whitney) for non-normally distributed variables. Linear correlation between variables was tested using Pearson for normally distributed and Spearman *r* for non-normally distributed variables. The significance level was set at *p* < 0.05.

Data Availability

Any data not published within the article because of space limitations will be shared as anonymized data by request from

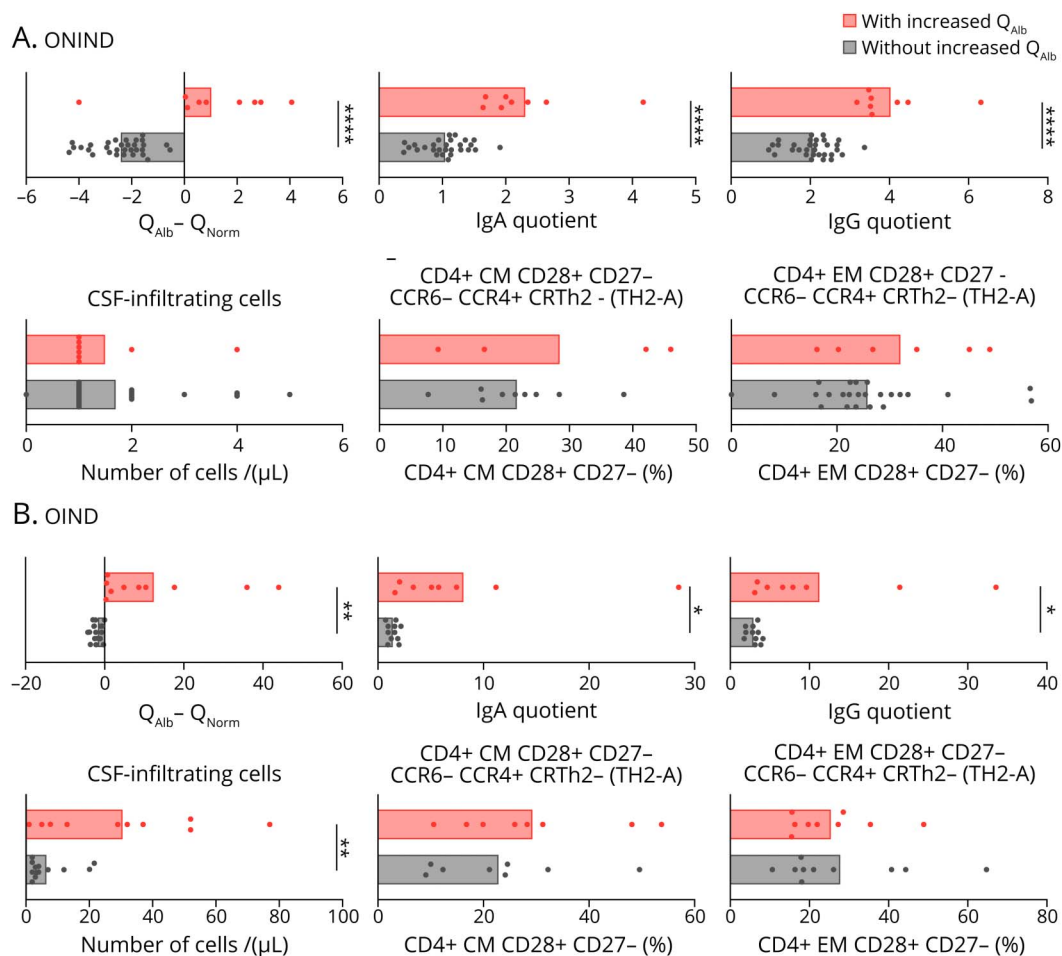
any qualified investigator for purposes of replicating procedures and results.

Results

Patients With MS With Increased Q_{Alb}

We determined the Q_{Alb} in our cohort of 121 patients with MS, and, assuming that this parameter increases with age, we marked a maximum Q_{Norm} for each patient as explained in M&M. We defined patients with increased Q_{Alb} as patients having a Q_{Alb} > Q_{Norm}. We used increased Q_{Alb} as indicator of BBB permeability although we are aware that it is not a perfect measurement because other factors such as altered CSF flow due to meningeal inflammation might influence it.¹⁵ Based on this definition of increased Q_{Alb}, 30 (24.8%) of patients with MS had an increased Q_{Alb} and 91 (75.2%) not (figure 1A). Demographic and clinical features of these patients are summarized in table 1. None of these parameters showed significant differences between patients with

Figure 6 Characterization of CSF-infiltrating Cells in Controls With and Without Increased Q_{Alb}



(A and B) Q_{Alb} minus Q_{Norm} has been used like in patients with MS to differentiate controls with (in red) and without (in black) increased Q_{Alb}. Comparison between controls (ONIND [n = 40] (A) and OIND [n = 21] (B)) with and without increased Q_{Alb} of IgA and IgG quotient, CSF-infiltrating cells, and frequency of TH2-A (CCR6- CCR4+ CRTh2-) and TH2-B (CCR6- CCR4+ CRTh2-) in CD4⁺ CM CD28⁺ CD27- and in CD4⁺ EM CD28⁺ CD27- CSF-infiltrating cells. In all graphs, each dot corresponds to 1 patient, and bars show the mean. T test *t* test for normally distributed variables and U test (Mann-Whitney) for non-normally distributed variables was used to compared patients. Statistical significance (**p* < 0.05, ***p* < 0.01, ****p* < 0.001, and *****p* < 0.0001) is shown. Q_{Alb} = serum albumin quotient; Q_{Norm} = normal Q_{Alb}.

MS with and without increased Q_{Alb}. HLA typing of these patients demonstrated that the expression of the MS-associated DR15 haplotype was also comparable in both groups of patients (table 1). We did not find any HLA class I or class II molecule over- or under-represented in patients with increased Q_{Alb} (data not shown).

CSF Measures in Patients With MS With Increased Q_{Alb}

IgA quotients were much higher in patients with MS with increased Q_{Alb}, IgG quotients were also significantly higher in these patients although to a lesser extent, and minimal differences were found for IgM quotients (figure 1B). In contrast, the indices for these 3 Igs corrected for albumin concentration (see M&M) were comparable in both groups of patients suggesting a strong association between the traffic of albumin and IgA and to a lesser extent IgG (table 1). Regarding the Igs(loc), only IgG(loc) was significantly lower

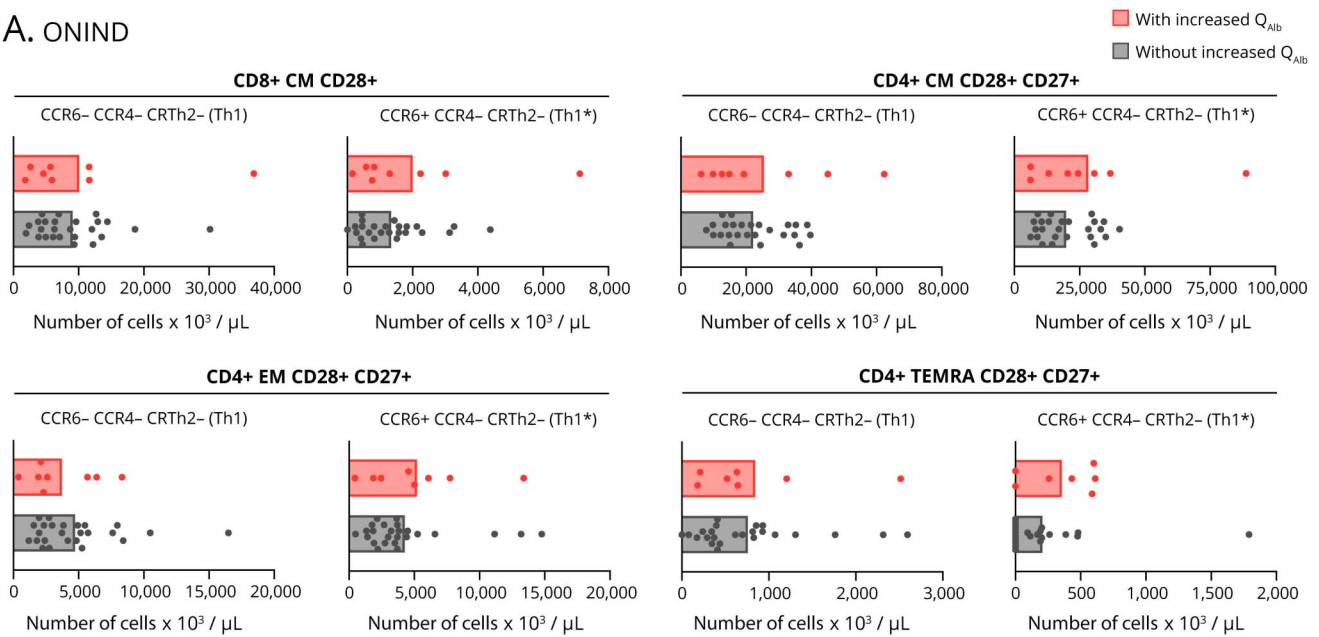
in patients with increased Q_{Alb} (table 1 and figure 1B). Total CSF protein was significantly higher in patients with MS with increased Q_{Alb}, whereas serum albumin, intrathecal lactate, and glucose quotient were comparable in patients with and without increased Q_{Alb} (table 1).

CSF-Infiltrating Cells in Patients With MS With Increased Q_{Alb}

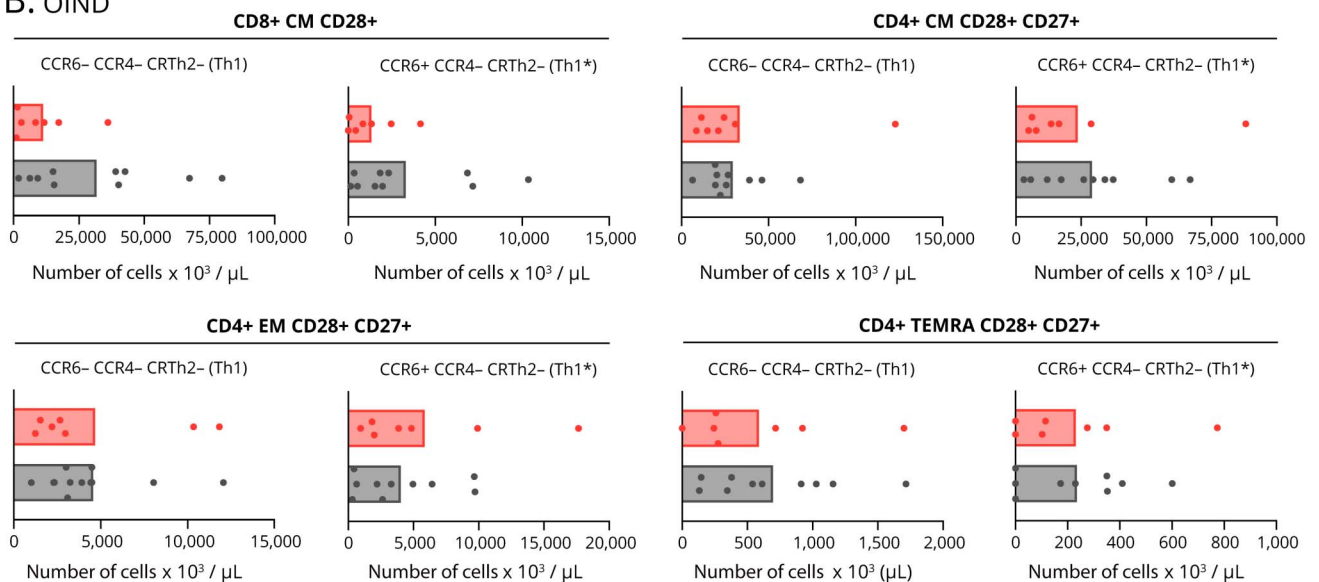
CSF analysis under the microscope showed comparable numbers of CSF-infiltrating cells in patients with and without increased Q_{Alb}, but different composition. CSF from patients with increased Q_{Alb} contains less lymphocytes but significantly more monocytes (figure 1C). This different cell composition of the CSF might underlie the lower correlation between IgG(loc) and number of CSF-infiltrating cells in patients with increased Q_{Alb} (figure 1D). Flow cytometry immunophenotyping of CSF and paired blood from a subcohort of 79 patients with MS (table 1, subcohort 1)¹² allowed a detailed characterization of

Figure 7 Characterization of Peripheral Circulating Cells in Controls With and Without Increased QAlb

A. ONIND



B. OIND



(A and B) Comparison between controls (ONIND [n = 40] (A) and OIND (n = 21) (B)) with and without increased QAlb of number of: CD8⁺ CM CD28⁺ Th1 (CCR6⁻ CCR4⁻ CRTh2⁻) and Th1* (CCR6⁺ CCR4⁻ CRTh2⁻), CD4⁺ CM CD28⁺ CD27⁺ Th1 (CCR6⁻ CCR4⁻ CRTh2⁻) and Th1* (CCR6⁺ CCR4⁻ CRTh2⁻), CD4⁺ EM CD28⁺ CD27⁺ Th1 (CCR6⁻ CCR4⁻ CRTh2⁻) and Th1* (CCR6⁺ CCR4⁻ CRTh2⁻), and CD4⁺ TEMRA CD28⁺ Th1 (CCR6⁻ CCR4⁻ CRTh2⁻) and Th1* (CCR6⁺ CCR4⁻ CRTh2⁻) circulating cells. Cell counts have been determined using SPHEROTM AccuCount Particles. In all graphs, each dot corresponds to 1 patient, and bars show the mean. T test t test for normally distributed variables and U test (Mann-Whitney) for non-normally distributed variables was used to compared patients.

CSF-infiltrating and circulating lymphocytes (table e-1, links. www.NXI/A413). Although most of the CSF-infiltrating cells in patients with and without increased QAlb showed similar frequencies, we found that CD4⁺ T cells with a Th2-A functional phenotype, based on the combination of chemokine receptors (CCR6⁻ CCR4⁺ CRTh2⁻), and the following different maturation states: (1) CM and EM CD28⁺ CD27⁻ CD4⁺ T cells, that have downregulated CD27 most likely

following repetitive stimulation with antigen,¹⁶ (2) EM CD28⁻ and (3) TEMRA CD28⁺ CD27⁺, were all significantly reduced in patients with increased QAlb (table e-1, figure e-2A, and figure 2A). Patients with MS with increased QAlb showed lower levels of these Th2 CD4⁺ T cells than patients without increased QAlb independently of disease activity and most likely clinical form of the disease (data not shown). Th2 cells are required for humoral immunity, and accordingly, we found

a significant association between the frequencies of EM CD28⁺ CD27⁻ CD4⁺ Th2-A cells and IgG(oc) (%) (figure 2B). Patients with increased QAlb also showed significantly higher frequencies of EM CD28⁻ TH1 cells as well as significantly higher amounts of IFN γ in the CSF that correlated with QAlb (figure 2C). None of the other 12 cytokines showed differences between patients with and without increased QAlb (figure e-2B). The frequencies of these cell types in peripheral blood were comparable between patients with and without increased QAlb (table e-1 and figure e-2C).

Markers of CNS Damage in Patients With MS With Increased QAlb

Because previous studies associated increased QAlb with higher disability in patients with MS,^{7,8} we explored indicators of CNS damage in our cohort of 121 patients with MS using 2 promising biomarkers: NF-L, a cytoskeletal component of neurons that is very abundant in axons,¹⁷ and it has been associated with BBB integrity,¹⁸ and CHI3L1, a glycoprotein secreted by activated glia.¹⁹ Our results demonstrated that the intrathecal quantity of both biomarkers was statistically higher in the CSF of patients with increased QAlb (figure 2D), and their amount correlated with QAlb and with each other as already reported²⁰ (figure 2E). The higher amount of NF-L and CHI3L1 in patients with MS with increased QAlb does not seem to be related to disease activity or clinical form of the disease (data not shown), although the low number of patients with radiologically isolated syndrome/clinically isolated syndrome and progressive MS forms limits conclusions.

Next, we used liquid chromatography/mass spectrometry to analyze the lipidomic profile in a subcohort of 36 patients with MS (table 1, subcohort 2). Because the brain has the second highest lipid content after adipose tissue, lipids are the main components of myelin sheaths and changes in lipid metabolism have been observed in damaged cells, we used lipidomics to explore CNS damage. The total lipid content in CSF reflected about 0.1%–0.5% of the plasma levels. In total, we identified 510 different lipid species in CSF of which 105 passed the QC criteria ($R^2 > 0.9 + CV < 20\%$). The intrathecal concentrations of 10 species were significantly higher in the CSF of patients with increased QAlb (figure 3A). The majority of these species showed a significant correlation with QAlb, whereas the strongest association was seen for the phosphatidylcholines (PCs) PC (38:3) and PC (40:5) and the sphingomyelins (SMs) SM (d40:3), SM (d41:2), SM (d42:2), and SM (d42:3) (figure 3B). Comparing the lipid profile in the CSF from patients with MS with the plasma levels from an independent reference cohort ($n = 32$) showed a distinct CSF/plasma ratio for the individual species indicating that this increased in the intrathecal lipids was selective and not simply caused by diffusion from plasma (figure 3C).

Peripheral Inflammation in Patients With MS With Increased QAlb

Because previous studies associated increased QAlb with systemic inflammation in patients with abnormal CSF measures,⁶ we quantified peripheral blood lymphocytes in MS subcohort 1

(table 1) using flow cytometry immunophenotyping and counting beads. Although the frequencies of the lymphocyte subtypes did not show significant differences between patients with and without increased QAlb, the real counts of several of these cells did (table e-1, links.lww.com/NXI/A413). Patients with increased QAlb had significantly higher counts of circulating CD3⁺ T cells, CD8⁺, and in particular CD4⁺ cells (table e-1). For each T-cell type, particularly CM and EM cells were increased (table e-1). Regarding the functional phenotype of these T cells, CM CD28⁺ CD8⁺ T cells and CM, EM, as well as TEMRA CD28⁺ CD27⁺ CD4⁺ T cells, all with Th1 (CCR6⁻ CCR4⁻ CRTh2⁻) and Th1* (CCR6⁺ CCR4⁻ CRTh2⁻) functional phenotypes were significantly increased in the blood of patients with increased QAlb (figure 4). In addition, a statistically significant correlation between the number of these T-cell subtypes in blood and the QAlb was observed (figure 4).

Disease activity and the clinical form of the disease does not seem to influence the higher frequency of these cells in patients with MS with increased QAlb (data not shown).

Increased Albumin Quotient Is Preserved Over Time

CSFs from 33 patients with MS were analyzed at different time points. All patients were untreated at the first time point. Eleven patients (33.3%) had increased QAlb at the first time point and 10 of these patients (83.3%) maintained increased QAlb over time, in 2 patients for even more than 40 months (figure 5A). Six of these patients received treatment between lumbar punctures (1 dimethyl fumarate, 3 fingolimod, and 2 natalizumab), whereas 4 patients remained untreated. The increased QAlb disappeared only in 1 patient after 58 months without treatment. Of the 22 patients without increased QAlb at the first time point, only 2 showed increased QAlb in subsequent analyses (figure 5A). Although some treatments can have an effect on BBB permeability and reduced QAlb values, our results suggest that in patients with increased QAlb the reduction to normal QAlb values is not easy and, if possible, it might take time.

We analyzed the number of CSF-infiltrating cells in the same samples. Fifteen patients (45.45%) had more than 4 CSF-infiltrating cells per μ L at the first time point, but only 6 of these patients (40%) maintained elevated CSF cell counts over time and no patient for more than 20 months (figure 5B).

Regarding the 16 patients with low CSF cell counts at the first time point, only 2 patients showed increased numbers over time (figure 5B).

Increased QAlb in ONIND and OIND

Finally, we examined whether the differences in CSF-infiltrating and circulating lymphocytes between patients with MS with and without increased QAlb were also present in controls (table 1). Using the definition of increased QAlb as $QAlb > Q_{Norm}$, we identified 9 (22.5%) ONIND and 10 (47.6%) OIND patients with increased QAlb and 31 (77.5%) ONIND and 11 (52.4%)

OIND patients without (figure 6, A and B). As observed in patients with MS, all controls with increased QAlb showed significantly higher intrathecal IgA and IgG (figure 6, A and B). The number of CSF-infiltrating cells was comparable between ONIND controls with and without increased QAlb, whereas it was significantly higher in OIND controls with increased QAlb (figure 6, A and B). The differences in CSF-infiltrating and peripheral blood lymphocytes that we observed in patients with MS with and without increased QAlb were not found in controls (figure 7, A and B).

Discussion

Different studies^{2–6} have suggested that CNS damage may increase the susceptibility of the BBB to changes induced by systemic inflammation such as increased vesicular trafficking of albumin. MS is a complex immune-mediated disease that affects the CNS and BBB permeability.²¹ In some patients with MS, CNS damage and systemic inflammation may co-exist and influence BBB function. With the aim to better understand BBB permeability in MS and to investigate a putative role of CNS damage and systemic inflammation on BBB dysfunction, we identified patients with MS with increased QAlb and characterized them in detail. The comparison of demographic and clinical features between patients with MS with and without increased QAlb did not reveal any statistically significant difference, although the limited clinical data did not allow a definitive statement. Unfortunately, neither longitudinal MRI data nor longitudinal disability measures were available from our cohort to confirm the previously reported association between increased QAlb and greater deterioration in patients with MS.^{7,8}

However, the detailed analysis of CSF revealed interesting differences between patients with and without increased QAlb. Patients with MS with increased QAlb showed significantly higher intrathecal levels of IgA and IgG and to a lesser extent of IgM. Because the indices of IgA and IgG, in which the values are corrected for the traffic of albumin, were comparable between patients, our results suggest that the passage of IgA and IgG across the BBB is most likely associated with the passage of albumin. IgM with its pentameric nature, much larger size, and molecular weight may not use vesicular trafficking. Patients with MS with increased QAlb also showed reduced IgG(10c). Regarding the CSF infiltrate, although the number of infiltrating cells was comparable in patients with and without increased QAlb, the cellular composition was different. Patients with MS with increased QAlb contained significantly more monocytes.

Because monocytes are not involved in antibody production, the correlation between CSF-infiltrating cells and IgG(10c) was significantly lower in patients with increased QAlb. Of interest, mediators involved in BBB dysfunction such as reactive oxygen species²² and matrix metalloproteinase 9²³ are produced by monocytes. Our *ex vivo* immunophenotyping of CSF-

infiltrating lymphocytes demonstrated that patients with MS with increased QAlb and reduced IgG(10c) also showed significantly lower frequencies of CD4⁺ T cells with a Th2 functional phenotype. In particular, the following CD4⁺ T cell subsets: CM and EM CD28⁺ CD27[−] CD4⁺ T cells, which may be relevant due to their downregulation of CD27 after repetitive stimulation with antigen,¹⁶ EM CD28[−] and TEMRA CD28⁺ CD27⁺ CD4⁺ T cells. Th2 cells are relevant for humoral immunity, and accordingly, we demonstrated an association between these cells and intrathecal IgG synthesis. Th2 cells, in addition to their role in humoral immunity, may also have a protective anti-inflammatory role as has been assumed in some MS clinical trials aimed to deviate immune responses from Th1 toward Th2.²⁴ Their reduction in patients with MS with increased QAlb might thus have harmful consequences. We also found that EM CD28[−] CD4⁺ T cells with a Th1 functional phenotype were significantly higher in patients with increased QAlb as well as the amount of IFN γ in the CSF. Long-term memory CD4⁺ CD28[−] T cells can produce high amounts of IFN γ in the absence of costimulation and showed increased survival and proliferation capacity after activation.²⁵ Their putative role in MS is supported by their expansion in a subgroup of patients with MS and their myelin basic protein specificity.²⁶ Immunophenotyping of paired blood samples did not show the same differences that have been observed between patients by CSF analyses. It is therefore unlikely that mere bystander traffic is responsible for the changes in the CSF. The higher concentration of IgG and IgA observed in patients with MS with increased QAlb, as well as the higher frequency of monocytes, the higher amount of IFN γ and the lower frequency of potentially protective Th2 CD4⁺ T cells might facilitate CNS damage in these patients. Accordingly, our results demonstrate that patients with MS with increased QAlb show significantly higher levels of biomarkers of CNS damage and inflammation such as NF-L and CHI3L1 and support previous studies, in which increased QAlb in patients with MS was found to be associated with atrophy and greater deterioration^{7,8} as well as higher levels of serum NF-L.¹⁸ In addition, we identified 10 intrathecal lipid species that are more abundant in patients with MS with increased QAlb.

Although we do not know the underlying mechanisms, the increase of these lipids may be involved in CNS damage. Two of the 4 identified SM species contained a Sphingadienine (SAdienine) long chain base, which is formed as a downstream product of sphingosine via fatty acid desaturase 3 (FADS3).²⁷ The increase in SAdienine-based SM suggests a possible role of FADS3 in this process. Of interest, a previous study linked changes in the same SAdienine-based SM species to an increased activity of acid sphingomyelinase, which converts SM to ceramide.²⁸ The activation of the acid sphingomyelinase/ceramide system has been demonstrated to be a central step for the development of EAE.²⁹

Regarding a putative association between systemic inflammation and BBB permeability in patients with MS, our results demonstrate that several lymphocyte populations, in

particular T cells with a Th1 and Th1* functional phenotype, were significantly more abundant in patients with MS with increased QALb and cell counts correlated with QALb. Of interest, this association was not observed in controls patients with ONIND and OIND diseases, suggesting that the type of systemic inflammation that might influence BBB permeability most likely differs between pathologies. Unfortunately, the usual and easy-to-determine markers of systemic inflammation such as C-reactive protein or neutrophil counts were not available from the blood taken at the time of lumbar puncture.

The coexistence of markers of CNS damage and systemic inflammation in patients with MS with increased QALb supports a putative role of CNS damage in increasing BBB vulnerability to systemic inflammation in these patients. Because the capacity of remyelination is limited and neuroregenerative treatments are missing, we can assume that CNS damage might persist over time. Accordingly, increased QALb in patients with MS was also preserved over time even after initiating immunomodulatory treatments. It will be interesting to see in the future if treatments or treatment combinations with neuroregenerative potential have an effect on QALb. Finally, given that CNS damage and systemic inflammation vary among patients with MS and influence BBB permeability, also it will be of interest to examine their impact on MS heterogeneity and its value in patient classification. Patients with increased QALb might especially profit from a better control of systemic inflammation as well as from neuroprotective/neuroregenerating treatment approaches.

Study Funding

European Research Council Advanced Grant (340733) (R.M.), Clinical Research Priority Program MS (CRPPMS) and (CRPP) PrecisionMS of the University Zurich (R.M., M.S., and A.L.), Clinical, Swiss National Science Foundation (Sinergia UnmetMS) (R.M. and M.S.), and the Swiss MS Society (R.M.).

Disclosure

The authors declare that they have no competing financial interests in the context of this work. Go to Neurology.org/NN for full disclosures.

Publication History

Received by *Neurology: Neuroimmunology & Neuroinflammation* June 3, 2020. Accepted in final form October 6, 2020.

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Marco Puthenparampil, MD, PhD	University Hospital and University Zurich, Switzerland; University Hospital of Padova, Italy	Major role in the acquisition of data and revised the manuscript for intellectual content
Paula Tomas-Ojer	University Hospital and University Zurich, Switzerland	Major role in the acquisition of data

Appendix (continued)

Name	Location	Contribution
Thorsten Hornemann, PhD	University Hospital and University Zurich, Switzerland	Acquisition of data and revised the manuscript for intellectual content
Andreas Lutterotti, MD	University Hospital and University Zurich, Switzerland	Revised the manuscript for intellectual content
Ilijas Jelcic, MD, PhD	University Hospital and University Zurich, Switzerland	Revised the manuscript for intellectual content
Mario Ziegler	University Hospital and University Zurich, Switzerland	Acquisition of data
Andreas J. Hülsmeier, PhD	University Hospital and University Zurich, Switzerland	Acquisition of data and revised the manuscript for intellectual content
Carolina Cruciani, PhD	University Hospital and University Zurich, Switzerland	Acquisition of data
Wolfgang Faigle, PhD	University Hospital and University Zurich, Switzerland	Revised the manuscript for intellectual content
Roland Martin, MD, PhD	University Hospital and University Zurich, Switzerland	Interpreted the data and revised the manuscript for intellectual content
Mireia Sospedra, PhD	University Hospital and University Zurich, Switzerland	Designed and conceptualized the study; analyzed the data; interpreted the data; and drafted the manuscript for intellectual content

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Neurol Neuroimmunol Neuroinflamm 2021;8;
DOI 10.1212/NXI.0000000000000951

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